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PATENT & TRADEMARK OFFICE

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s)	: James Harrison Aylward	Docket	: 14923Z
Serial No	: 09/888,178	Group Art Unit	: 1654
Filed	: June 21, 2001	Examiner	: Christopher Robin Tate
For	: ANTI CANCER COMPOUNDS		

Commissioner of
Patent and Trademarks
Washington, D.C. 20231

DECLARATION PURSUANT TO 37 C.F.R. §1.132

I, Dr. James Harrison Aylward, hereby declare as follows:

1. I am currently the Research Director of Peplin Operations Pty Ltd, a subsidiary of Peplin Biotech Ltd, Ground Floor, South Tower, 527 Gregory Terrace, Bowen Hills, Brisbane, QLD, 4006, Australia. My Curriculum Vitae is attached hereto as Exhibit **JHA-1**.

2. I have published extensively in the area of biochemistry. A list of my publications is included in my Curriculum Vitae (Exhibit **JHA-1**).

3. I am an inventor of subject matter contained and described in United States Patent Application Serial No. 09/888,178 filed on 21 June, 2001 (hereinafter referred to as the "APPLICATION"). The APPLICATION is directed *inter alia* to a method for treating cancer by administering to the subject in need thereof a therapeutically effective amount of an angeloyl-substituted ingenane obtainable from the sap of a

Euphorbia species and an active derivative of an angeloyl-substituted ingenane obtainable from the sap of a *Euphorbia* species.

An example of a derivative of an angeloyl-substituted ingenane is an acetylated derivative. Acylation of the free accessible hydroxyls on ingenane 8 and 9 should improve their stability by preventing acyl migration. A number of acyl groups could be chosen, but as a test system, acetylation was selected. The chemical structure of ingenane 8 and 9 are shown in Exhibit **JHA-2**

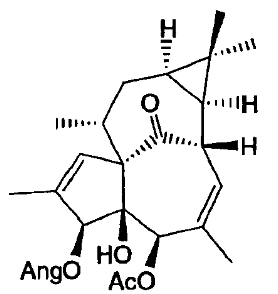
4. Acyl migration is probably an intramolecular process involving attack of a free hydroxyl group on a closely situated carbonyl carbon. The developing positive charge on the attacking oxygen and the developing negative charge on the carbonyl oxygen are likely to be more stabilized in more polar solvents and thus this process is more likely to be observed in these. In the peplus milk the non-polar diterpenes and associated latex may form vesicles with non-polar interiors which act to protect the molecules from the polar aqueous environment, but these would be broken down on purification leaving the compounds more susceptible to such processes.

5. In conjunction with my scientific collaborators, I have conducted routine experiments in the acetylation of Ingenanes, the experimental details which follow acetylated derivatives of angeloyl –substituted ingenanes were tested for anticancer activity. All had strong bipolar activity of at least 1000 bipolar units, as measured by reversion of malignant melanoma MM96L cells to a bipolar dendritic morphology, the assay as described in United States Patent Application Serial No. 09/888,178 filed on 21 June, 2000.

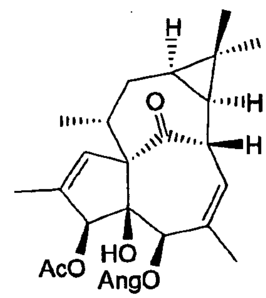
Example 1:

Acetylation of a 6mg mixture of ingenane 8 and its 5-angeloyl isomer arising from acyl migration in ingenane 8, was carried out with acetyl chloride in dry pyridine to give a mixture which contained two major components identified as the 3-O-angelate-

5-O-acetate and the 5-O-angelate-3-O-acetate in quantities of 1.7 and 2.3mg (ca 50% combined yield). These had bipolar activity at about the same level as ingenane 8 and its 5-angeloyl isomer.



3-O-angelate-5-O-acetate



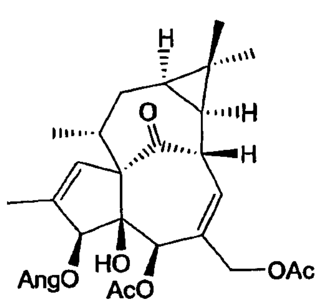
5-O-angelate-3-O-acetate

The experimental details of the acetylation of ingenane 8 are as follows.
3-angeloyloxy-4,5-dihydroxyingena-1,6-dien-9-one (6mg) was stood in methanol/water for several days at -4°C then concentrated, dissolved in anhydrous pyridine (100µl) and treated with acetyl chloride (10µl)at room temperature for 24h. Water (1ml) was added and the resulting mixture passed through a 7mm diam. x 20mm column of Chromatorex ODS resin (Fuji Silysia Chemical Co.). The resin was washed successively with water (7ml), 1:1 water:methanol (8ml) and methanol (36ml). The combined methanol eluates were concentrated and subjected to HPTLC on Merck 10 x 20cm HPTLC plate coated with LiChrospher Si60F_{254s} (eluent 50% MTB in 40-60° bp petroleum spirit). Concentration of the ether extract of the excised band with R_f 0.95 gave a colourless gum (4.4mg) which was subjected to HPLC on a 10mm diam. x 250mm Alltima C18-5u column with 68% methanol in water, isocratic for 40 minutes then a non-linear gradient to 100% methanol over 118 mins.
Concentration of the eluate containing the peak at 165 mins. gave 5-acetoxy-3-angeloyloxy-4-hydroxyingena-1,6-dien-9-one (compound I) as a colourless gum (2mg). APCIMS$^+$ m/z 479 (7) $[M+Na]^+$, 457 (4) $[M+H]^+$, 397 (3) $[M-OAc]^+$, 357 (8) $[M\text{-angelate}]^+$, 315 (17) $[M\text{-angelate}, -AcOH]^+$, 297 (100) $[M\text{-angelate}, -AcOH, -H_2O]^+$, 269 (19) $[M\text{-angelate}, -H_2O, -CO]^+$.

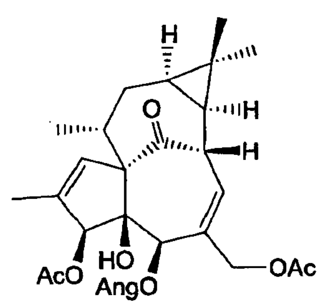
Concentration of the eluate containing the peak at 153 mins. gave a mixture which was resubjected to HPLC on a 10mm diam. x 250mm Alltima C18-5u column with 80% methanol in water, isocratic for 64 mins. then a non-linear gradient to 100% methanol over 21 mins. Concentration of the eluate containing the peak at 52 mins. gave 3-acetoxy-5-angeloyloxy-4-hydroxyingena-1,6-dien-9-one (compound II) as a colourless gum (2mg). APCIMS$^+$ m/z 397 (6) $[M\text{-}OAc]^+$, 315 (50) $[M\text{-angelate, -AcOH}]^+$, 297 (100) $[M\text{-angelate, -AcOH, -}H_2O]^+$, 269 (29) $[M\text{-angelate, -}H_2O\text{, -CO}]^+$ (see Tables I, II and III for details).

Example 2:
Acetylation of a 10mg mixture of ingenane 9 and its isomers arising from acyl migration in ingenane 9, was carried out similarly to give a mixture which contained two major components identified as the 3-O-angelate-5,20-O-diacetate and the 5-O-angelate-3,20-O-diacetate in quantities of 0.9 and 1.2mg (ca 19% combined yield).

These had bipolar activity at about tenfold more than the ingenane 8 derivatives and it did not appear to vary significantly between the two isomers.



3-O-angelate-5,20-O-diacetate



5-O-angelate-3,20-O-diacetate

The experimental details of the acetylation of ingenane 9 are as follows.
3-angeloyloxy-4,5,20-trihydroxyingena-1,6-dien-9-one (10mg) was stood in methanol/water for several days at -4°C then concentrated, dissolved in anhydrous pyridine (200µl) and treated with acetyl chloride (20µl)at room temperature for 24h. Water (1ml) was added and the resulting mixture passed through a 7mm diam. x

20mm column of Chromatorex ODS resin (Fuji Silysia Chemical Co.). The resin was washed successively with water (7ml), 1:1 water:methanol (8ml) and methanol (36ml). The combined methanol eluates were concentrated and subjected to HPTLC on Merck 10 x 20cm HPTLC plate coated with LiChrospher Si60F_{254s} (eluent 50% MTB in 40-60° bp petroleum spirit). Concentration of the ether extract of the excised band with R_f 0.89 gave a colourless gum (3.2mg) which was subjected to HPLC on a 10mm diam. x 250mm Alltima C18-5u column with 80% methanol in water, isocratic for 102 mins. then a linear gradient to 100% methanol over 14 mins.

Concentration of the eluate containing the peak at 52 mins. gave 3-angeloyloxy-5,20-bis(acetoxy)-4-hydroxyingena-1,6-dien-9-one (compound III) as a colourless gum (1mg). $APCIMS^+$ m/z 537 (13) $[M(C_{29}H_{38}O_8)+Na]^+$, 515 (3) $[M+H]^+$, 313 (22) $[M\text{-angelate, -angelic acid, -}CH_2CHO]^+$, 295 (100) [M-angelate, -angelic acid, $-CH_2CHO, -H_2O]^+$.

Concentration of the eluate containing the peak at 30 mins. gave 5-angeloyloxy-3,20-bis(acetoxy)-4-hydroxyingena-1,6-dien-9-one (compound IV) as a colourless gum (1mg). $APCIMS^+$ m/z 537 (13) $[M(C_{29}H_{38}O_8)+Na]^+$, 497 (6) $[M-OH]^+$, 455 (9) $[M-AcO]^+$, 313 (19) $[M\text{-angelate, -angelic acid, -}CH_2CHO]^+$, 295 (100) [M-angelate, -angelic acid, $-CH_2CHO, -H_2O]^+$ (see Tables I, II and III for details).

Note that the lower yield in this case is probably due to the other isomers which were produced in small quantities but not isolated. In both cases the yields are probably significantly lower than might be expected on a larger scale due to the problems with handling such small quantities (see Tables I, II and III for details).

These acetylated compounds appeared to be more stable than ingenane 8 and ingenane 9.

Table I. [1]H NMR data (CD_2Cl_2, 500MHz) for compounds I-IV

	δ (ppm)			
H	I	II	III	IV
1	6.05 q	6.06 bs	6.05 q	6.07 bs
3	5.01 bs	4.97 bs	5.06 s	4.99 bs
5	5.21 bs	5.33 bs	5.37 bs	5.47 bs
7	5.83 dq	5.83 dq	6.22 bd	6.21 bd
8	4.17 bd	4.19 bd	4.24 bdd	4.25 bd
11	2.51 ddq	2.51 ddq	2.54 ddq	2.55 ddq
12	2.29 ddd	2.30 ddd	2.27 ddd	2.28 ddd
12'	1.72 ddd	1.75 ddd	1.75 ddd	1.79 ddd
13	0.67 ddd	0.68 ddd	0.71 ddd	0.72 ddd
14	0.86 dd	0.87 dd	0.92 dd	0.92 dd
16	1.06 s	1.08 s	1.07 s	1.09 s
17	1.04 s	1.05 s	1.05 s	1.06 s
18	0.95 d	0.96 d	0.97 d	0.98 d
19	1.75 d	1.75 d	1.76 d	1.76 d
20	1.55 s	1.53 s	4.57 bd	4.47 bd
20'			4.19 d	4.20 d
3-OAng 2'-Me	1.89 dq		1.89 dq	
3-OAng 3'	6.13 qq		6.14 qq	
3-OAng 4'	1.97 dq		1.97 dq	
5-OAng 2'-Me		1.99 dq		1.97 dq
5-OAng 3'		6.23 qq		6.24 qq
5-OAng 4'		2.01 dq		2.01 dq
3-OAc				2.09 s
5-OAc	2.26 s		2.22 s	
20-OAc			1.98 s	1.94 s
4-OH	3.31 bs	3.13 bs	3.36 bs	
	J (Hz)			
J 1,19	1	1	1.4	1.4
J 7,8	5	5	5	5
J 7,20	1.4	1.4		
J 8,14	12	12	12	12
J 11,12	4	3	3	3
J 11,12'	4	5	5	5
J 11,18	7	7	7	7
J 12,12'	16	16	16	16
J 12,13	10	10	10	9
J 12',13	7	6	6	7
J 13,14	8	8	8	8
J 20,20'			12	13
OAng J2'-Me,3'	1.4	1	1.4	1.4
OAng J2'-Me,4'	1.4	1.4	1.8	1.4
OAng J3',4'	7	7	7	7

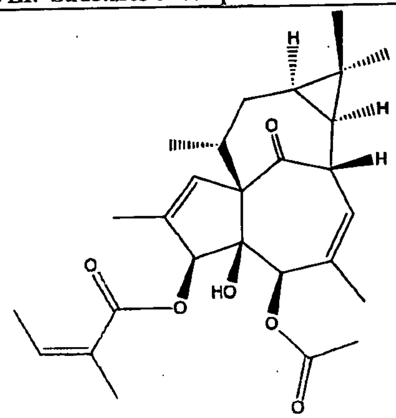
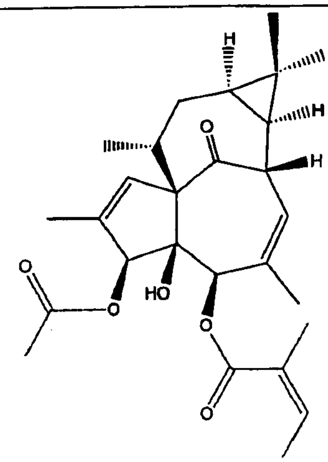
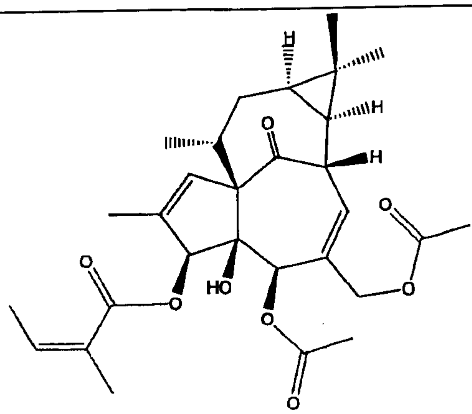
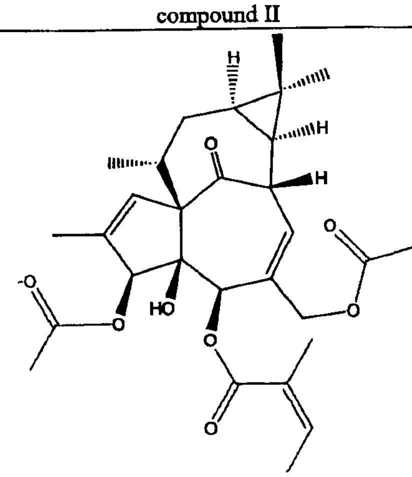
Table II. ^{13}C NMR data (CD_2Cl_2, 125MHz) for compounds I-IV

	δ (ppm)			
C	I	II*	III	IV
1	132.8	132.9	132.5	132.7
2	135.9	136.2	136.3	135.8
3	82.6	83.0	82.2	82.7
4	86.3	86.3	86.4	86.4
5	78.0	78.1	75.4	74.4
6	135.1	135.0	134.0	132.7
7	128.1	126.4	132.1	131.8
8	43.9	43.9	44.2	43.6
9	206.2	#	205.8	206.7
10	72.4	72.4	72.6	72.6
11	39.2	39.1	39.1	39.5
12	31.4	31.4	31.5	31.5
13	23.5	23.5	23.6	23.6
14	23.7	23.8	23.5	23.4
15	24.8	24.8	24.8	24.8
16	15.8	15.8	15.8	15.8
17	28.7	28.7	28.7	28.7
18	17.1	17.2	17.2	17.3
19	15.7	15.7	15.7	15.7
20	21.5	21.7	66.3	66.6
3-OAng 1’	169.4		169.4	
3-OAng 2’	126.7		127.9	
3-OAng 2’-Me	21.0		21.0	
3-OAng 3’	139.3		139.7	
3-OAng 4’	16.1		16.2	
5-OAng 1’		167.1		166.3
5-OAng 2’		127.6		127.3
5-OAng 2’-Me		20.8		20.7
5-OAng 3’		141.1		142.1
5-OAng 4’		16.3		16.3
3-OAc 1’		172.7		171.9
3-OAc 2’		21.5		21.5
5-OAc 1’	170.5		170.5	
5-OAc 2’	21.2		21.2	
20-OAc 1’			170.2	170.0
20-OAc 2’			21.0	21.1

* incomplete spectrum

unable to determine value

Table III: Structures of compounds I-IV

 compound I	 compound II
 compound III	 compound IV

Indications are that these compounds as expected are more stable than ingenanes 8 and 9, though 3-acetoxy-5-angeloyloxy-4-hydroxyingena-1,6-dien-9-one still has some stability problems.

6. It is my considered scientific opinion that these data support the claim that cancer can be treated by administering to the subject in need thereof a therapeutically effective amount of an angeloyl-substituted ingenane obtainable from the sap of a *Euphorbia* species and an active derivative of an angeloyl-substituted ingenane obtainable from the sap of a *Euphorbia* species.

The undersigned declares further that all statements made herein are of his own knowledge, are true, and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful, false statements, and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code.

Date: August 22 2003

Dr. James Harrison Aylward

EXHIBIT JHA-1

CURRICULUM VITAE

JAMES HARRISON AYLWARD

Home Address:	14 Marston Ave Indooroopilly, QLD, 4068 Australia Phone: +617 3371 9287
Present Work Location:	G floor Comprehensive Cancer Research Centre Queensland Institute of Medical Research The Bancroft Centre Royal Brisbane Hospital Herston, Brisbane, QLD, 4006 Australia
Date of Birth:	July 1, 1948, Springvale, Victoria, Australia
Present Position:	Research Director Peplin Operations Pty Ltd, a subsidiary of Peplin Biotech Ltd Ground Floor, South Tower 527 Gregory Terrace Bowen Hills, Brisbane, QLD, 4006 Australia Phone: +617 3854 0980 Fax: + 617 3854 0989 Mobile: +61419 710 808 Email: jim.aylward@peplin.com
Marital Status:	Married, no children

Formal Education:

1972-75	PhD (Biochemistry) Monash University, Clayton, Victoria, Australia
1970-71	MSc qualifying (Biochemistry), Monash University, Clayton, Victoria, Australia
1967-69:	BSc, majors in Chemistry & Biochemistry, Monash University, Clayton, Victoria, Australia
1966:	Matriculation, Huntingdale High School, Huntingdale, Victoria, Australia

Professional Experience:

April 1998 – present time	*Research Director, Peplin Biotech* direction of research relating to commercialisation of novel small molecules with biological activity, with focus on anticancer activity. Co-founder of Peplin Biotech in 1998
1992 - April 1998:	*Principal Research Scientist* CSIRO Division of Tropical Agriculture 306 Carmody Road, St. Lucia, QLD 4068, Australia Project Leader 1993-95 (Biotechnology group) Budget responsibility: AUD $1.5m pa improving the nutrition of ruminants by increasing the nutritive value of dietary fibre by manipulation of enzymes of fibre degradation in the rumen, using the tools of protein biochemistry and molecular biology enzymes for use in the paper pulp industry use of bacteria and yeasts as biocontrol agents for protection of fruits and vegetables from fungal spoilage agents for use in opportunistic fungal infections and as immune system boosters anti-cancer compounds which promote cellular differentiation development of new functional foods DNA incorporation into bacteria using sub micron gold particles
1984-91	*Senior Research Scientist/Principal Research Scientist* CSIRO Division of Tropical Animal Production, Meiers Road, Indooroopilly, QLD, 4068, Australia vaccines against tick-borne diseases
1981-83	*Research Scientist/Senior Research Scientist* CSIRO Division of Tropical Crops and Pastures, Cunningham Laboratory, St, Lucia QLD, 4068, Australia

nutritive value and toxicity testing of new dietary legumes (beans) for ruminants and monogastrics

1980-81

Senior Tutor
Monash University, Department of Biochemistry, Clayton, VIC, 3168, Australia

control of intermediary metabolism by fragments of growth hormone in muscle, adipose tissue and liver

1979-80

Research Associate
Department of Physiology
Howard Hughes Medical Institute
Vanderbilt University, Nashville, Tennessee, USA

mechanism of insulin and adrenalin action on muscle glycogen synthase, a key enzyme in control of carbohydrate metabolism

1976-78

Research Associate
Department of Biochemistry
University of Miami School of Medicine
Miami, Florida, USA

enzymology of phosphorylase phosphatase, a key enzyme in energy metabolism under hormonal control

Publications

Patent applications (CSIRO owned)

Inventors: **Aylward, J.H.** and Stone. B.F. (1991) "Tick paralysis toxin" *Australia 86784*

Inventors: Aylward, J.H. and Orpin, C.G. (1992) "Biocontrol bacteria" *Australia PL 0256*

Inventors: Williamson, M.A. and **Aylward, J.H.** (1992) "Biocontrol agents for use in horticulture" *Australia PL 8298*

Inventors: **Aylward, J.H.,** Riddles, P.W., and Wright, I.G. (1993) "Antigens and polypeptides derived from Babesia (12D3) antigen." *Australia 640398*

Inventors: **Aylward, J.H.** and Williamson, M.A. (1993) "Biocontrol agents for use in agricultural products" *Australia PL 7721*

Inventors: Xue, G-P., Gobius, K.S., **Aylward, J.H.**, and Orpin, C.G. (1993) "Recombinant cellulases"

Inventors: **Aylward, J.H.**, and Williamson, M.A. (1996) "Biocontrol agents in treatment of opportunistic infections" *Australia PN 9072*

Non-CSIRO owned

Inventor: **Aylward, J.H.** (1997) "Anti-cancer compounds" *Australia Provisional PO 8640,* PCT/AU98/00656 (transferred to Peplin Biotech Pty Ltd)

Papers and Book chapters

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Aylward, J.H., Bornstein, J., Gould, M.K. and Hall, S. (1974) Inhibition of muscle pyruvate dehydrogenase by a polypeptide from growth hormone. *Biochemical Biophysical Research Communications* **59** 57-62.

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Lee, E.Y.C., Mellgren, R.L., Killilea, S.D. and **Aylward, J.H.**(1978) Properties and regulation of liver protein phosphatases. In *"Regulatory mechanisms of carbohydrate metabolism" (Ed V. Esmann) FEBS Symposium* **42** 327-346 (Pergamon Press, New York).

Lee, E.Y.C., **Aylward, J.H.**, Mellgren, R.L., and Killilea, S.D. (1979) Protein phosphatase C: properties, specificity and structural relationship to a larger holoenzyme. In: *"From gene to protein: information transfer in normal and abnormal cells"* (Eds. T.R. Russell et al), pp. 483-500 (Academic Press, new York).

Mellgren, R.L., **Aylward, J.H.**, Killilea, S.D. and Lee, E.Y.C. (1979) The activation and dissociation of a high molecular weight form of rabbit skeletal muscle phosphorylase phosphatase by endogenous Ca^{2+}-dependent proteases. *Journal of Biological Chemistry* **254** 648-652.

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Aylward, J.H., Court, R.D., Haydock, K.P., Strickland, R.W. and Hegarty, M.P. (1987) Indigofera species with agronomic potential in the tropics: rat toxicity studies. *Australian Journal of Agricultural Research* **38** 177-86.

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Xue, G-P., Johnson, J.S., Smyth, D.J., Dierens, L.M., Wang, X., Simpson, G.D., Gobius, K.S. and **Aylward, J.H.** (1996) Temperature-regulated expression of the *tac/lac* system for overproduction of a fungal xylanase in *Escherichia coli. Applied Microbiology and Biotechnology* **45** *120-126.*

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Aylward, J.H., Xue, G-P., Simpson, G.D. and Orpin, C.G. (1993) Cellobiohydrolase (CBH) from *Neocallimastix patriciarum*: a membrane associated complex? In. *Proceedings of the 17th International Grassland Congress*, Palmerston North, New Zealand, February 8-21, 1993. New Zealand Grassland Association; pp.1222-1224.

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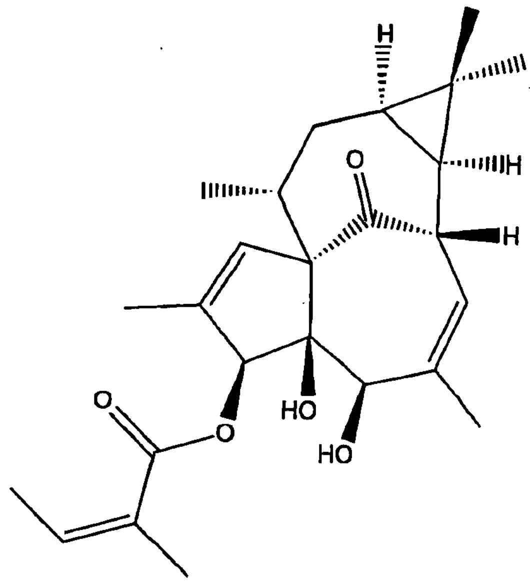
EXHIBIT JHA-2



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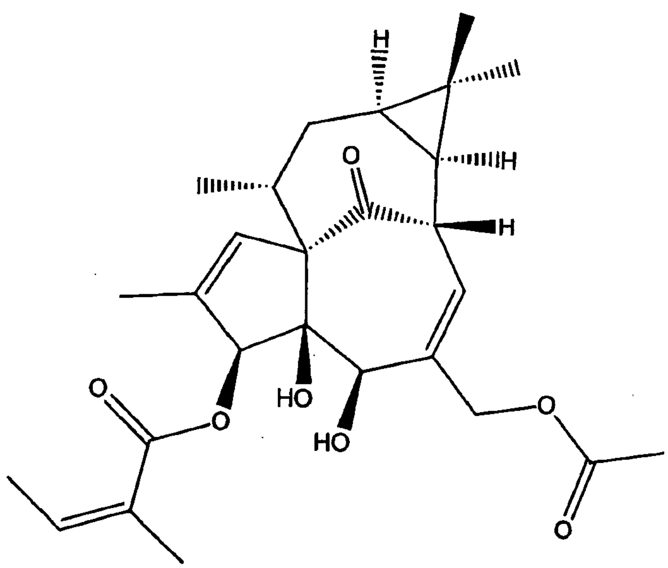

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